AD_____

Award Number: DAMD17-02-1-0509

TITLE: Role of p53 in Mammary Epithelial Cell Senescence

PRINCIPAL INVESTIGATOR: Goberdhan Dimri, Ph.D.

CONTRACTING ORGANIZATION: New England Medical Center Hospitals, Incorporated
Boston, Massachusetts 02111

REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2004	Annual (1 May 2003 - 30 Apr 2004)

4. TITLE AND SUBTITLE
Role of p53 in Mammary Epithelial Cell Senescence

5. FUNDING NUMBERS
DAMD17-02-1-0509

6. AUTHOR(S)
Goberdhan Dimri, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Medical Center Hospitals, Incorporated
Boston, Massachusetts 02111

E-Mail: gdimri@enh.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The tumor suppressor p53 plays an important role in a variety of cancers including breast cancer. It inhibits the growth of malignant cells either by inducing G1 arrest, apoptosis or senescence. We are determining the role of p53 in human mammary epithelial cell (HMEC) senescence. We previously showed that p53 protein level do not change in senescence of pre-selection HMECs, however p53 and its target gene p21 are significantly upregulated during senescence in post-selection HMECs. Do further study the role of p53 in senescence of postselection HMECs, we used knockdown approach using RNA interference (RNAi) technology. We generated post-selection HMECs stably expressing p53 and p21 RNAi. Here we show that cells with reduced p53 or p21 proteins have extended replicative life span. At the end of life span cells expressing p53 or p21 RNAi entered a crisis like stage without any emergence of immortal clones. Furthermore, we show that compared to p21 RNAi, p53 RNAi expressing cells proliferates for four more population doublings. Our data suggest that other transcriptional targets of p53 may also be involved in replicative senescence of post-selection HMECs. We are using chromatin immunoprecipitaion linked PCR (ChIP) assay to identify these additional targets of p53 involved in replicative senescence of HMECs.

14. SUBJECT TERMS
Cell proliferation, senescence, tumor suppressor, p53, HMECs

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

In most cases, breast cancer is a carcinoma arising from the transformation of mammary epithelial cells. Transformation is a complex multistep process involving several molecular genetic changes. It is believed that the first molecular genetic change entails bypass of cellular senescence followed by the immortalization of cells (1, 2). After completing a certain number of divisions, normal cells enter a state of irreversible growth arrest and altered function, known as cellular senescence (3). Multiple lines of evidence suggest that cellular senescence constitute a tumor suppressive mechanism (4, 5). In somatic cells, telomerase remains repressed and telomere length keeps shortening at each round of DNA replication. Short telomeres signal cells to stop further proliferation and invoke a permanent growth arrest phenotype known as replicative senescence (6, 7). Two important tumor suppressor pRb and p53 are required for the maintenance and genesis of senescent phenotype.

p53 is widely considered a cellular gatekeeper, and mutations in p53 allow cells to accumulate genetic abnormalities and become cancerous (8). The p53 protein is a typical transcription factor and contains an N-terminus transactivation, a centrally located DNA binding and a C-terminus oligomerization domains (8). Transcriptionally active p53 binds to a consensus site 5' -RRRCA/TA/TYYY-3', often present in pairs in p53 regulated genes (8). Tumor derived mutants of p53 are always defective in sequence-specific transactivation, thus attesting the importance of transcription activation function of p53. In response to various physiological stimuli, p53 undergoes post-translational modifications such as phosphorylation and acetylation, which activate p53 transcription functions (8, 9). Activation of these transcription activation functions results in either apoptosis, G1 and G2 cell cycle arrest or senescence (8-10).

When mammary tissue is explanted in an appropriate tissue culture medium, a heterogeneous cell population emerges. This heterogeneous population proliferates for 3-5 population doublings before a majority of cells undergoes senescence. Regular feeding of these cells (sometimes) give rise to a homogeneous population which is referred to post-selection HMECs, while the original heterogeneous mixture is referred to as pre-selection cells (11, 12). Senescence in pre-selection cells, which is also termed as M0 stage, appears to be due to the accumulation of p16 (13, 14), and emergence of post-selection homogeneous culture is believed to arise due to progressive methylation of p16 locus (11-14). However, post-selection cells still undergo senescence and never spontaneously immortalize. In the previous report, we showed that p53 and p21 is significantly upregulated during senescence in post-selection HMECs but not in pre-selection HMECs. To further understand the role of p53 and its target genes in senescence of post-selection HMECs, we used RNA interference (RNAi) approach (15).

RNAi technology is being widely used to downregulate various endogenous proteins and generate loss of function phenotype in mammalian cells (15-18).This technology is based on inhibitory function of double strand short inhibitory RNAs (shRNAs) or synthetic short interfering RNAs (siRNAs) These short double strand RNAs corresponding to a small stretch of mRNA sequence (usually 19-21 nucleotides) in a particular mRNA targets that mRNA to degrade via a complex but incompletely understood pathway (15). The stable expression of siRNAs has been achieved using an RNA Polymerase III dependent promoter of Histone H-1 (17, 18). A double strand oligonucleotide corresponding to 5' AA[N19-21] 3' in the mRNA of interest is cloned under the pol III promoter. After transcription in the cell, this construct generates a 19-21 bp stem and a short loop structure, which acts as a very efficient siRNA (17, 18). Using this system, downregulation (of protein and its function) of p53 and several other proteins has been demonstrated (17, 18).

BODY:

76N pre- and post-selection cells were obtained from Dr. Vimla Band. These cells were cultured in DFCI-1 medium as described (19). Cells were serially passaged in culture until senescence. Senescence was determined using senescence associated beta-galactosidase (SA-β-gal) assay and using ^{3}H-thymdine incorporation assay (% labeled nuclei or %LN) as described (19, 20). Cells were considered early passage when >70% cells incorporated ^{3}H-thymidine and less than 5% cells were SA-β-gal positive. Conversely cells were considered senescent when SA-β-gal index was >70% and %LN were 10-15%. SA-β-gal is a widely used senescence marker used in various cell types including HMECs. To knockdown p53 and p21 in HMECs, we used stable expression of p53 and p21 shRNA (short hairpin) using retrovirus. We obtained a plasmid vector containing p53 shRNA (20, 21) from Oligoengine Inc, Seattle, WA. p53 shRNA was subcloned in pRS (pRetroSuper) vector. We also generated a vector that expresses p21 RNAi. Retrovirus vector expressing p53RNAi, p21RNAi and a control RNAi, which contain p21 scrambled shRNA sequence were transfected into tsa54, a packaging cell line and retroviruses were generated as described (19, 20). The retroviruses were introduced into 76N cells to generate cells stably expressing p53 and p21 RNAi.

RESEARCH ACCOMPLISHMENTS

1. Generation of 76Np53i and 76Np21 RNAi cell lines-

76N cells were infected with these retrovirus vectors and selected in puromycin. Cells stably expressing p53shRNA, p21RNAshRNA and a control shRNA were further grown in culture. These cells lines are herein referred as 76Np53i, 76Np21i and 76Ncont.i respectively. Steady state level of p53 and p21 in selected cells were examined in these cell lines by western blot analysis using respective antibodies. The results (Fig.1) clearly demonstrated significant downregulation of p53 and p21 in 76Np53i and 76Np21i cells respectively. As expected p53 RNAi cells also have virtually undetectable levels of p21.

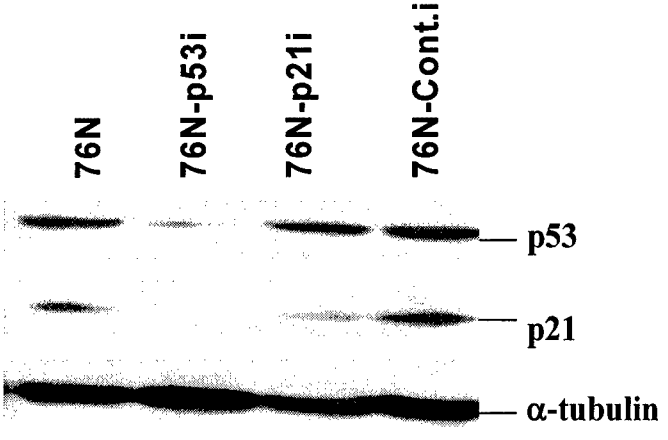

Figure 1: Downregulation of p53 and p21 in 76Np53i and 76Np21i cells respectively. Steady state level of p53, p21 and α-tubulin were determined in 76N, 76Np53i, 76Np21i, 76Ncont.i (as indicated) by western blot analysis as described (19, 20).

2. 76Np53i and 76Np21i cell line exhibit finite but extended replicative life span-

76Np53i, and 76Ncont.i and mock infected 76N cells were further passaged in culture. Cells were passaged in complete D (DFCI-1) as well as D2 (DFCI-2) medium that lacks growth factors and isused for the selection of life-span extended or immortalized clones (19). The replicative lifespan of these cells was studied as described. Results (table 1) showed that compared to mock and 76Ncont.i, 76Np53i and 76Np21i cells exhibited significant replicative life span extension. However, despite significant replicative life span extension, p53i and p21i cells are not immortal and cultures failed to yield any immortal clones suggesting abrogation of p53-p21 pathway is not sufficient for immortalization of HMECs. At the end of replicative life span, p53i and p21i cells underwent crisis and eventually cell death. The results also demonstrate that p53 abrogation is more effective in extending the replicative life span than p21 abrogation, suggesting the role of additional targets of p53 in senescence of HMECs.

Table 1

Cells	Cumulative Population Doublings (CPDs)*
76N Mock	6
76Ncont.i	5
76Np53i	30
76Np21i	26

*CPDs were determined in D2 medium. Cells after selection were considered at PD1.

KEY RESEARCH ACCOMPLISHMENTS:

During past two years, I have learned how to culture pre- and post-selection HMECs, and determine senescence in these cells. I have developed HMECs cell lines stable expressing p53 and p21 RNAi. These cells are further being characterized and used to study senescence in HMECs. I have also optimized p53 DNA binding and chromatin-immunoprecipitate linked PCR (ChIP) assay. The key research accomplishments during past year are following:

- p53 DNA binding activity increases with senescence in post-selection HMECs.
- p53 level and its transcription activity as determined by examining the level of its target gene p21 increases with senescence in post- but not pre-selection HMECs.
- There are no significant posttranslational changes in p53 during senescence in HMECs as determined by a limited set of antibodies.
- Stable downregulation of p53 and/or p21 using RNAi approach significantly extends replicative life span of HMECs
- Stable downregulation of p53 is more effective than p21 downregulation in extending the replicative life span of HMECs.

REPORTABLE OUTCOMES: None

CONCLUSIONS:

p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence. Gradual telomere shortening is thought to provoke a DNA damage checkpoint mediated by p53, which results in permanent growth arrest. Most tumor cells have lost this ability to undergo senescence and cycle even when telomere lengths critically short. In this report, we have presented evidence that p53 may play an important role in senescence of post-selection cells but not pre-selection cells.

In the first year of the grant, we proposed to study the DNA binding activity, its expression level and posttranslational modifications during senescence in HMECs. We have completed the proposed studies. However, we have not found any significant differences in posttranslational modifications using limited number of antibodies that we used.

In the second year, we started using p53 RNAi approach to study the role of p53 in senescence. We generated post-selection HMECs cells with p53 and p21 knockdown using RNAi approach. The study of replicative life span of these cells suggest that p53 plays an important role in senescence of post-selection HMECs and other target genes of p53 are possibly involved in senescence. Next year we plan to perform p53 ChIP analysis in post-selection HMECs and identify additional targets of p53 involved in HMEC senescence as previously proposed in the grant application.

REFRENCES:

1. Hanahan, D., and R. Weinberg. 2000. The hallmarks of cancer. *Cell* **100:** 57-70.
2. Hahn, W.C., C.M. Counter, A.S. Lundberg, R.L. Beijersbergen, M.W. Brooks, and R.A. Weinberg. 1999. Creation of human tumor cells with defined genetic elements. *Nature* **400:** 464-468.
3. Campisi, J. 1996. Replicative senescence: An old lives' tale? *Cell* **84:** 497-500.
4. Dimri G.P, and J. Campisi. 1995. Molecular and cell biology of replicative senescence. *Cold Spring Harbor Symposia on Quantitative Biology: Molecular Genetics of Cancer* **54:** 67-73.
5. Smith, J and Pereira-Smith, O. 1996. Replicative senescence: implications for *in vivo* aging and tumor suppression. Science. **273:** 63-67.
6. Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* **345:** 458-460.
7. Kim Sh SH, Kaminker P, Campisi J. Telomeres, aging and cancer: in search of a happy ending. Oncogene. 2002 Jan 21;21(4):503-11.
8. Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88:** 323-331.
9. Appella, E., and C.W. Anderson. 2001. Post-translational modifications and activation of p53 by genotoxic stresses. *Eur. J. Biochem.* **268:** 2764-72
10. Itahana, K., G. Dimri, and J. Campisi 2001. Regulation of cellular senescence by p53. *Eur. J. Biochem.* **268:** 2784-2791.
11. Ratsch, S.B., Q. Gao, S. Srinivasan, D. Wazer, and V. Band. 2001. Multiple Genetic Changes Are Required for Efficient Immortalization of Different Subtypes of Normal Human Mammary Epithelial Cells. *Rad. Res.***155:** 143-150.
12. Wong, D.J., S.A. Foster, D.A. Galloway, and B.J. Reid. 1999. Progressive region-specific de novo methylation of the p16 CpG island in primary human mammary epithelial cell strains

during escape from M(0) growth arrest. *Mol.Cell. Biol.* **8:** 5642-5651.

13. Brenner, A.J, M.R. Stampfer, and C.M. Aldaz. 1998. Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. *Oncogene* **17**: 199- 205.
14. Romanov, S.R., B.K. Kozakiewicz, C.R. Holst, M.R. Stampfer, L.M. Haupt, and T.D. Tlsty. 2001. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature* **409:** 633-637.
15. Hannon, G. J. (2002) RNA interference. *Nature* **418:** 244-251.
16. Elbashir, S. M, Harborth, J., Weber, K., and Tuschl, T. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26:** 199-213.
17. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550-553.
18. Brummelkamp, T., Bernards, R., and Agami, R. (2002) Stable suppression of tumorigenicity by virus- mediated RNA interference. *Cancer Cell* **2:** 243-247.
19. Dimri, G. P., Martinez, J.L., Jacobs, J.L, Keblusek, P., Itahana, K., van Lohuizen, M., Campisi, J. Wazer, D.E., and Band, V. (2002) Bmi-1 oncogene induces telomerase and immortalizes human mammary epithelial cells. *Cancer Res.* **62:** 4736-4745.
20. Itahana, K., Ying , Z., Itahana, Y., Martinez, J. L., Beausejour, C., Jacobs, J. L., van Lohuizen, M., Band, V., Campisi, J. and Dimri, G. P. (2003) Control of replicative senescence in human fibroblast by p16 and the polycomb protein Bmi-1. *Mol. Cell. Biol.* **23:** 389-401.